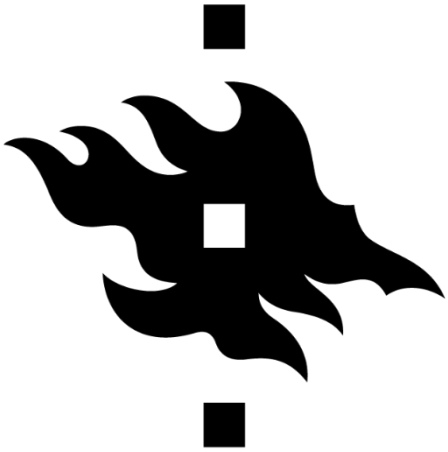
ASSESSING THE SITE-SPECIFICITY OF CANCER-PRECEDING GENE EXPRESSION CHANGES IN MOUSE COLON MUCOSA

Sonja Nykänen

Master's Thesis



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Tiivistelmä – Referat – Abstract

Colorectal cancer (CRC) kills more than half a million people a year worldwide. Usually the disease develops over several years via multiple steps which involve both genetic and epigenetic alterations. CRC is often diagnosed at late stage, when the cancer has already metastasized, and the prognosis is relatively poor. Several studies suggest that the first changes towards colorectal cancer occur and can be detected in histologically normal tissue before the appearance of any detectable lesion. The pre-cancerous cells harbouring those changes may form a field of tissue, which is predisposed to malignant transformation. The study of pre-cancerous tissue might reveal the earliest changes in CRC development, which can be used as biomarkers for early detection and prevention of CRC.

The aim of this thesis was to revise and investigate whether the aberrant expression of the six chromosomal segregation genes, *Bub1, Mis18a, Pms2, Rad9a, Tpx2, and Mlh1,* would signal carcinogenesis in mouse colon mucosa. Altogether fourteen mice, of which six had a proximal colon carcinoma, were selected for the study. The expression analysis was performed to histologically normal colon mucosa collected from the proximal and distal colon of each mice in order to investigate whether the possible pre-cancerous changes are found exclusively in the close proximity to the carcinoma. The expression was quantified with reverse transcription quantitative polymerase chain reaction (RT-qPCR).

No statistically significant gene expression differences were found between the carcinoma and control mice, indicating that the studied mice did not display cancer-preceding expression changes of the six studied genes in the carcinoma adjacent histologically normal colon mucosa. The results differed from the previously reported results, where the expressions of the six genes were found to be downregulated in the carcinoma adjacent mucosa. Here, the sample size was presumably not large enough to reveal statistically significant clustering of the expression patterns. However, *Bub1* seemed to have a downregulated trend in the carcinoma adjacent mucosa, which supports the previously suggested role of *Bub1* alterations in CRC initiation.

Avainsanat – Nyckelord – Keywords
Colorectal cancer, gene expression, RT-qPCR, carcinogenesis

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Selvitys syöpää edeltävien geenien ekspressiomuutosten paikkaspesifisyydestä hiiren paksusuolen limakalvolla

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Tiivistelmä – Referat – Abstract

Paksusuolisyöpään kuolee vuosittain yli puoli miljoonaa ihmistä. Syövän kehitys on yleensä vuosia kestävä monivaiheinen prosessi, johon liittyy sekä geneettisiä että epigeneettisiä muutoksia. Paksusuolisyöpä havaitaan usein vasta taudin myöhäisessä vaiheessa, kun syöpä on jo ehtinyt levitä elimistössä, ja ennuste on suhteellisen huono. Useat tutkimukset viittaavat siihen, että syövän kehitystä edeltävät muutokset tapahtuvat histologisesti normaalissa limakalvossa jo ennen makroskooppisesti havaittavan limakalvon muutoksen ilmaantumista. Solut, joissa nämä muutokset tapahtuvat, saattavat muodostaa limakalvolle syövän kehitykselle altistuneen alue
en. Syövälle altistuneen limakalvon tutkiminen voi paljastaa syövän kehitykseen liittyviä aikaisia muutoksia, joita voitaisiin käyttää ns. biomarkkereina osoittamaan kohonnutta syöpäriskiä ja näin ollen myös mahdollisesti estämään syövän kehitys.

Tämän tutkielman tarkoitus oli selvittää, osoittaako kuuden kromosomien segregaatioon liittyvien geenien, *Bub1*, *Rad9a*, *Tpx2*, *Mlh1*, *Pms2* ja *Mis18a*, epänormaali ilmentyminen syövän kehitystä hiiren paksusuolen limakalvolla. Yhteensä neljätoista hiirtä, joista kuudella oli proksimaalisen paksusuolen karsinooma, valittiin tähän tutkimukseen. Geenien ilmentyminen mitattiin histologisesti normaalilta limakalvolta kunkin hiiren proksimaalisesta ja distaalisesta paksusuolesta, jotta saataisiin selville, ovatko mahdollisesti syöpää edeltävät muutokset havaittavissa ainoastaan karsinooman läheisyydessä. Geenien ilmentymistasot mitattiin käyttäen reaaliaikaista käänteistranskriptio-polymeraasiketjureaktiota (RT-qPCR).

Karsinooma- ja kontrollihiirten välillä ei havaittu tilastollisesti merkitseviä ilmentymistason eroja, mikä viittaa siihen, että tutkittujen hiirten karsinooman viereisessä histologisesti normaalissa limakalvossa ei ollut syövälle altistavia tutkittujen geenien ilmentymisen muutoksia. Saadut tulokset eriävät aiemmista tutkimustuloksista, joissa näiden kuuden geenin ilmentyminen havaittiin olevan alentunut karsinooman viereisessä limakalvossa. Otoskoko ei oletettavasti ollut tässä tutkimuksessa riittävän suuri paljastamaan tilastollisesti merkitseviä eroja. Vaikka tilastollista merkitsevyyttä ei löytynyt, *Bub1*- geenin ilmentymistaso vaikutti olevan hieman laskenut karsinooman viereisessä limakalvossa, mikä tukee aiemmin ehdotettua *Bub1*- geenin muutosten osallisuutta syövän kehitykseen.

Avainsanat – Nyckelord – Keywords
Paksusuolisyöpä, geenien ilmentyminen, RT-qPCR, karsinogeneesi

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ABBREVIATIONS

AIN	AIN-93, American Institute of Nutrition, purified diet for laboratory rodents
bp	base pair
CD	distal colon
cDNA	complementary DNA
CENP-A	centromere protein A
CIN	chromosomal instability
CIMP	CpG island methylator phenotype
CPR	proximal colon
Cq	quantification cycle
CRC	colorectal cancer
FAP	Familial adenomatous polyposis
gDNA	genomic DNA
IBD	inflammatory bowel disease
LOH	loss of heterozygosity
LS	Lynch syndrome
Min	Multiple intestinal neoplasia
MMR	mismatch repair
mRNA	messenger RNA
MSI	microsatellite instability
RIN	RNA integrity number
RNA-seq	RNA sequencing
RT-qPCR	reverse transcription quantitative polymerase chain reaction
SAC	spindle assembly checkpoint
SD	standard deviation
WD	Western diet
wt	wild type

1. INTRODUCTION

Colorectal cancer (CRC) is a major public health issue being the third most common cancer in the world. The CRC incidence rates have been increasing significantly from the 1980s, and the increase is expected to continue in coming years (Kuipers et al., 2015). This is, presumably, caused by population aging and wide adoption of so-called Western diet and lifestyle, which are recognized as major risk factors for CRC (Brenner, Kloor, & Pox, 2014). Other risk factors for CRC are, for instance, old age, obesity and inherited predisposition (Mármol, Sánchez-de-Diego, Pradilla Dieste, Cerrada, & Rodriguez Yoldi, 2017).

The prevention of metastatic tumor development by early detection and treatment is of the utmost importance in cancer management. Accordingly, the prognosis of CRC is highly dependent on the stage of the disease at diagnosis as the 5-year survival rate is approximately 90% for localized CRC but decreases to less than 10% when distant metastases have developed (Coppedè, Lopomo, Spisni, & Migliore, 2014). Thus, there is an increasing interest to identify novel biomarkers that are present in CRC in the early stages of the disease development. Yet the earliest events in the malignant transformation of colon cells, which could be utilized for early detection and prevention of CRC development, remain to be elucidated.

CRC evolves by stepwise accumulation of genetic and epigenetic alterations which eventually lead to colonic cell proliferation and metastasis. The molecular basis of CRC is heterogeneous with different pathways leading to different phenotypes. The loss of genomic stability is a crucial feature of cancer development, and likely to drive tumorigenesis by accelerating the accumulation of mutations (Pino & Chung, 2010). The cells harbouring some, but not all features required for malignancy, are thought to form a pre-malignant field of histologically normal colon mucosa in which the cancer is likely to arise (Curtius, Wright, & Graham, 2018). The identification of early events in tumorigenesis and the assessment of the site specificity of these changes poses an interesting possibility for early detection and prevention of CRC development.

Several studies have demonstrated that cancer-preceding changes are already seen in histologically normal colon mucosa. Pussila et al. (2018) studied cancer associated changes in mouse colon and were able to identify several down-regulated tumor suppressor genes in normal tumor-adjacent mucosa. Furthermore, Suvi Rantamo (2017) assessed the locality of these gene expression changes

in her Master's thesis by analysing the expression profiles from mucosa samples collected further away from the tumor. Interestingly, gene expression changes seen in the tumor adjacent histologically normal mucosa were not observed in the mucosa further away from the tumor, suggesting the existence of a field of aberrant tissue that surrounds the tumor. However, Pussila et al. (2018) and Rantamo (2017) used different methods to analyse the gene expression, and therefore, the expression levels measured from the two studies are not comparable. The study presented here was carried out in order to revise the finding of cancer-preceding profile and investigate its site specificity in mouse colon.

2. LITERATURE REVIEW

2.1 Colorectal cancer (CRC)

2.1.1 Epidemiology

Colorectal cancer is the third most common cancer in men and second most common cancer in women worldwide (Bray, Ren, Masuyer, & Ferlay, 2012). It is one of the leading causes of cancer related mortality, with over 600,000 deaths each year (Ferlay et al., 2012). The burden of CRC is not equally distributed across the world (Fig. 1). The incidence rates show wide geographical variation, and there is up to 10-fold difference between countries with the highest and lowest incidence (Haggar & Boushey, 2009).

CRC is mainly a disease of developed countries as about two-thirds of the cases occur in countries characterized by high or very high indexes of development and/or income (Bray, Ren, Masuyer, & Ferlay, 2012; Ferlay et al., 2012). The highest incidence rates are found in Australia, New Zealand, and Europe and the lowest rates in Africa and South-Central Asia (Ferlay et al., 2012). The so-called Western lifestyle and consumption of Western-style diet is recognized as a risk factor for CRC and is likely to explain the geographical and socio-economical differences in CRC incidence rates (Brenner, Kloor, & Pox, 2014; Kuipers et al., 2015).

The CRC mortality rates show less regional variation than the incidence rates (Fig. 1). Since the CRC survival is highly dependent on the stage of disease at diagnosis, the poor access to early detection and treatment is likely to explain the reduced survival in less developed countries (Arnold et al., 2017). Furthermore, the incidence and mortality rates seem to be stabilizing or decreasing in highly developed countries but rising in developing countries (Arnold et al., 2017). The changes are likely caused by improving cancer diagnosis and treatment in high-income countries, in contrast to developing countries, where the ongoing socio-economic development is presumably driving population towards more Western-style diet and lifestyle (Arnold et al., 2017). However, it should be noted that significant part of CRC cases might stay unreported in low-income countries, which may bias the global distribution.

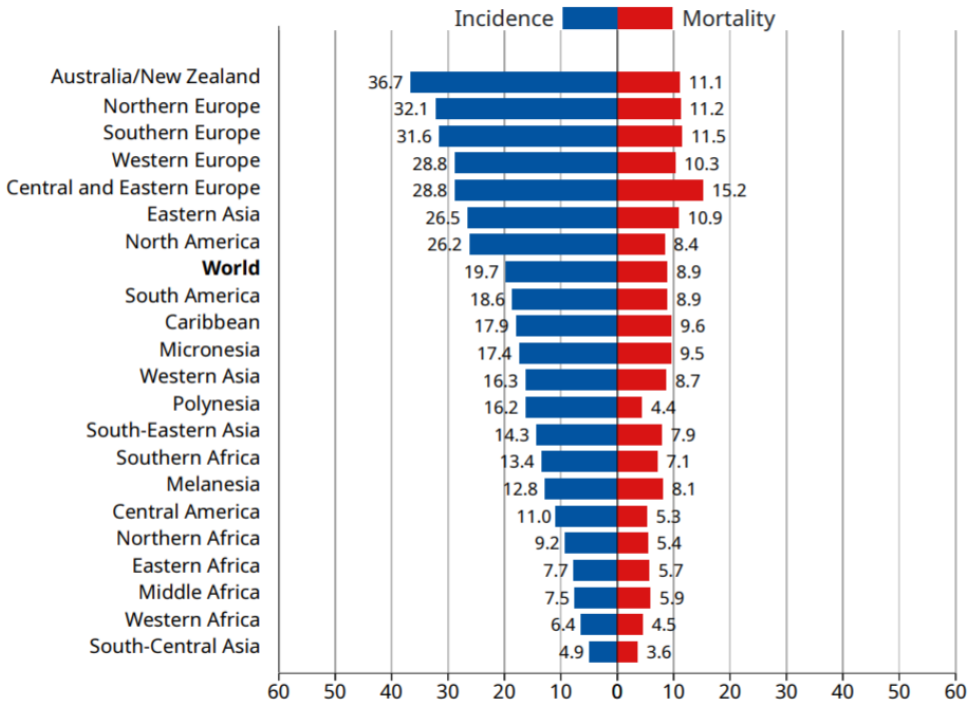

Fig. 1. Age-standardized global incidence and mortality rates per 100 000 individuals. The CRC incidence rates vary internationally, with the highest rates in Australia, New Zealand, and Europe and the lowest rates in Africa and South-Central Asia. Mortality rates show less geographical variation than incidence rates. The improved cancer diagnosis and treatment methods have presumably reduced the mortality in high-income countries. Modified from Ferlay et al. (2018).

2.1.2 Risk factors

Age is a major risk factor for colorectal cancer. The incidence is low at ages younger than 50, but the risk increases significantly after that (Mármol et al., 2017). In developed countries, the median age at diagnosis is 70 years (Brenner et al., 2014). Aging is defined as time-dependent functional decline, which is most likely a consequence of accumulated somatic damage partly due to age-related increase in mutation rate and decrease in DNA repair efficiency (Milholland, Auton, Suh, & Vijg, 2015). Accumulation of somatic mutations together with age-related epigenetic changes are the main factors contributing to increased CRC risk in elderly (Ahuja, Li, Mohan, Baylin, & Issa, 1998; Gorbunova, Seluanov, Mao, & Hine, 2007).

Along with aging, certain lifestyle factors, especially the lack of physical activity and consumption of Western diet, are associated with increased CRC risk. Western diet typically contains refined grains, processed meat, saturated fat and sugar, and excess of salt (Niederreiter, Adolph, & Tilg,

2018). Western diet is suggested to promote chronic inflammation and alter the gut microbiota, which are linked to the development of CRC (Niederreiter et al., 2018). The lack of physical activity and excessive calorie intake are associated with obesity, which is also an important risk factor for CRC. The high levels of body fat accelerate the chronic inflammation in colon and rectum, thereby increasing CRC risk (Mármol et al., 2017). Chronic inflammation can also be caused by inflammatory bowel disease (IBD). When compared to general population, patients with IBD are at higher risk of developing CRC, and the risk increases with the duration of the inflammation (Munkholm, 2003). Furthermore, the CRC risk can be reduced by physical activity and possibly by consuming diet rich in vitamin C and D, vegetables, fibre, whole grains, dairy products and fish (Table 1.) (World Cancer Research Fund & American Institute for Cancer Research, 2008).

Approximately one third of CRC patients have positive family history of the disease, suggesting an inherited susceptibility (Mármol et al., 2017). Familial cases, where the cancer predisposing component is not identified, account for approximately 25% of all CRCs (Mármol et al., 2017). A combination of environmental factors and low-penetrance genes is likely involved in the increased CRC risk in most of the familial cases (Kuipers et al., 2015). However, approximately 5% of CRC patients have hereditary colorectal cancer syndrome, where a high-penetrance germline mutation causes the increased risk (Kuipers et al., 2015). Depending on the mutation, lifetime risk of developing cancer may increase close to 100% in persons with inherited cancer syndrome if no medical or surgical intervention is performed (Dunlop, 2002). The discovery of genetic basis of hereditary CRC syndromes have provided crucial insights into molecular mechanisms of sporadic CRC. The most common syndromes are Lynch syndrome (LS) and Familial Adenomatous Polyposis (FAP), accounting for approximately 3% and 1 % of all CRCs, respectively (Brenner et al., 2014).

Lynch syndrome is an autosomal dominant condition caused by a germline mutation in one of the DNA mismatch repair (MMR) genes, most commonly in *mutL Homolog 1 (MLH1)* or *mutS Homolog 2 (MSH2),* and less frequently in *mutS Homolog 6* (*MSH6)* or *postmeiotic segregation increased 2* (*PMS2)* (Lynch et al., 2009). MMR genes are tumor suppressors, which are silenced when both alleles are inactivated. Since LS patients inherit one inactive allele, a somatic alteration that inactivates the other allele is enough to disrupt the MMR system. Defective MMR system leads to the accumulation of mutations, and therefore, predisposes to cancer. LS patients have significantly increased risk of developing CRC. The average age of onset in LS-associated CRC is low, 45 years, compared to the average age of onset in general population, which is 65 years (Lynch et al., 2009). Other characteristics of LS-associated CRC are rapid carcinogenesis, microsatellite instability (MSI) and

tumor tendency to locate in the proximal part of the colon (Lynch et al., 2009). Patients with LS are also at high risk for certain extracolonic cancers, such as stomach, ovary, biliary tract, urinary tract, pancreatic, small bowel and brain (Watson et al., 2008).

Germline mutation in *Adenomatous polyposis coli* (*APC)* is the cause of FAP. Similarly as in LS, a somatic mutation in the second allele triggers the FAP-associated cancer development. The most common mutation reported in FAP is a nonsense mutation in *APC* gene resulting in truncating protein (Galiatsatos & Foulkes, 2006). FAP patients develop numerous lesions predominantly in the distal colon, which almost inevitably progress to CRC by the age of 40 if left untreated (Galiatsatos & Foulkes, 2006).

Table 1. Summary of the factors contributing to colorectal cancer risk. Modified from Brenner et al. (2014).

Non-modifiable factors	
Older age	↑↑↑
Family history	↑↑
Inflammatory bowel disease	↑↑
Modifiable factors	
Smoking	↑
Excessive alcohol consumption	↑
High consumption of red and processed meat	↑
Obesity	↑
Physical activity	↓
Fruits and vegetables	(↓)
Cereal fibre and whole grain	(↓)
Fish	(↓)
Dairy products	(↓)

↑↑↑ = very strong risk increase, ↑↑ = strong risk increase, ↑ moderate risk increase, ↓ = moderate risk reduction. Parentheses show probable but not fully established associations.

2.1.3 Tumor development

Colon mucosa consists of crypts, which are tube-like glands made up of a single sheet of cells (Humphries & Wright, 2008). Replicating stem cells reside at the bottom of the crypt producing differentiating cells that migrate upwards until they reach the top of the crypt and undergo apoptosis (Kwong & Dove, 2009). There has been considerable debate about the events taking place when normal colon mucosa transforms towards cancer. The fact that intestinal epithelium is rapidly self-renewing has led to the assumption that long-lived stem cells are the only cells persisting long enough to gain successive mutations required in carcinogenesis (Huels & Sansom, 2015). This is consistent with the bottom up-hypothesis, which suggest that stem cells gain the initial mutations, expand and migrate upwards from the bottom of the crypt, which leads to the polyp formation at the top of the crypt (Fig. 2a) (Preston et al., 2003). Despite the wide acceptance of the bottom-up hypothesis, studies suggest that stem cells are not the only possible cells of origin in CRC (Davis et al., 2015; Hassan & Howell, 2000; Schwitalla et al., 2013). Bottom up hypothesis is challenged by the top-down hypothesis, first proposed by Shih et al. (2001). According to the top-down model, differentiated cells in upper part of the crypt re-acquire stem cell- like properties and form a dysplastic epithelium that extends laterally and downward towards the bottom of the crypt (Fig. 2b). To date, it seems likely that both stem and non-stem cells are capable of acquiring the initial mutations and start the transformation towards cancer. In fact, a recent study by Yi Hong et al. (2018) provided evidence for bidirectional initiation of colon cancer suggesting that top-down and bottom-up models for cancer initiation are equally likely.

In order to expand in colorectal epithelia, the mutant cell lineage has to colonize the neighbouring crypts. In top-down model, proliferating cells at the top of the crypt spill over and invade the neighbouring crypts, eventually replacing the normal mucosa (Shih et al., 2001). Crypt fission is an alternative way for mutant cells to expand in colorectal epithelia (Fig. 2c). Colonic crypts divide by crypt fission, which is essential for normal colon development (Humphries & Wright, 2008). Crypt fission occurs also in adult intestinal epithelium, but the frequency is low; crypt fission is estimated to take place every 112 days in mouse and every 17 years in human (Bjerknes, 1986; Humphries & Wright, 2008). Since the crypt fission is a relatively rare event, it would not seem very efficient mechanism for mutant cell expansion. However, mutations that occur early in tumorigenesis are suggested to accelerate the crypt fission, which would make it a potential mechanism for lateral expansion during tumorigenesis (Curtius et al., 2018).

Eventually, the abnormally proliferating cells form a histologically detectable mucosal aberration. Benign colonic lesions, termed polyps, are relatively common in humans, yet they only seldom progress to malignancy (Kwong & Dove, 2009). Adenomatous polyps are the most common mucosal aberrations that have the potential to progress to cancer (Rex et al., 2012). They are classified into three major histological groups according to their microscopic appearance; tubular, villous and tubulo-villous adenomas (Tanaka, 2009). Another type of cancer precursor is a serrated polyp, which is histologically characterized by a sawtooth appearance (Rex et al., 2012). Serrated polyps occur primarily in the proximal colon and are often challenging to detect due to their flat or sessile appearance (Hetzel et al., 2010). The process where normal colon mucosa transforms into malignant tumor usually takes over 10 year (Kuipers et al., 2015). However, in some situations, for instance in Lynch syndrome (LS) where the defective DNA repair leads to higher mutation rate, the process of tumor development is accelerated (Lynch et al., 2009). A regular colonoscopy, where the polyps can be detected and removed, is recommended for LS patients, which is indeed shown to reduce the risk of CRC (Järvinen et al., 2000; Lynch et al., 2009).

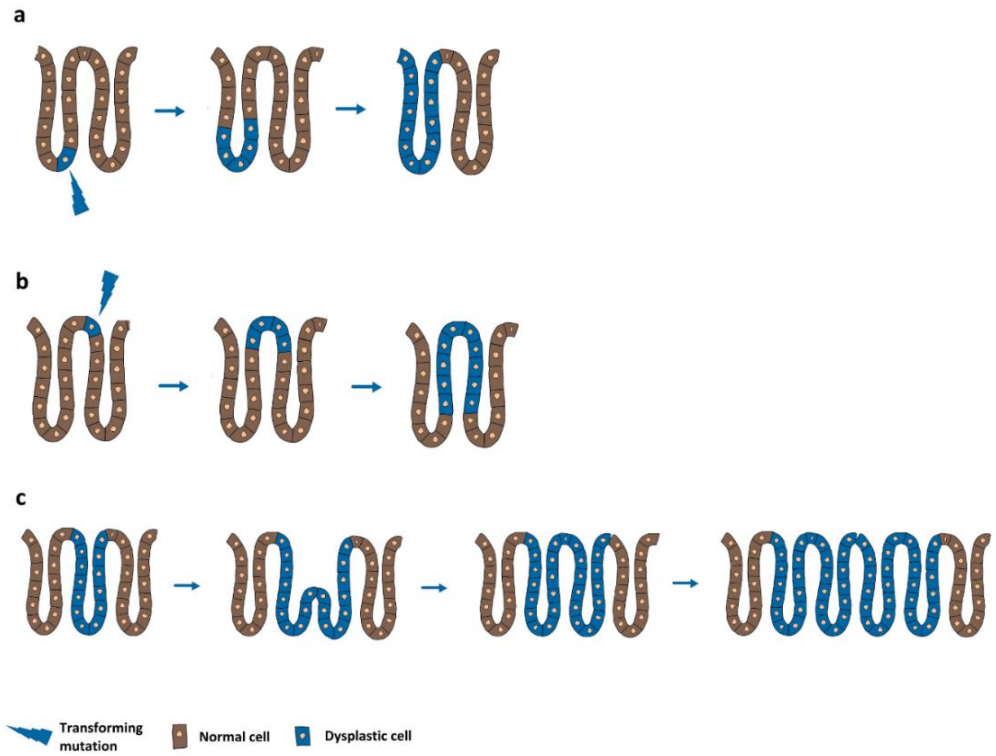

Fig. 2. Tumorigenesis in colon. A. Mutagenic insult in the crypt stem cell results in expanding mutant cell population and migration upwards to the top of the crypt (bottom-up hypothesis). B. As a consequence of mutagenic insult, differentiated cells in the top of the crypt re-acquire stem cell like properties, expand and migrate downward towards the bottom of the crypt (top-down hypothesis). C. Colonic crypts divide by crypt fission, which is a potential mechanism for lateral expansion of mutant cells. Modified from Greaves et al. (2006).

2.1.4 Molecular pathogenesis

Cancer arises through a heterogeneous process, where the accumulation of somatic mutations and epigenetic alterations eventually disrupt the normal cell behaviour (Greenman et al., 2007). Somatic mutations are commonly classified as driver and passenger mutations. Driver mutations confer selective advantage on the cell in which they occur, thus promoting cancer development (Greenman et al., 2007). The growth or survival advantage conferred by driver mutations together with favourable microenvironment enables the cell clone to outcompete neighbouring cells and expand in the tissue (Greaves & Maley, 2012). Passenger mutations do not confer fitness advantage, instead they arise in cells that harbour advantageous mutations, and become frequent by hitchhiking (Beerenwinkel et al., 2007). Cancer genomes often harbour alterations in numerous genes (Hanahan & Weinberg, 2011). The most critical regulatory genes altered in cancer are traditionally classified as oncogenes and tumor suppressor genes. Oncogenes drive cell proliferation as a consequence of activating alterations. Tumor suppressor genes inhibit tumor development by restraining inappropriate cell proliferation, stimulating apoptosis and maintaining the DNA sequence (Hanahan & Weinberg, 2011).

The adenoma-carcinoma sequence, introduced by Fearon and Vogelstein in the beginning of the 90's, is still widely accepted model for colorectal tumorigenesis (Fearon & Vogelstein, 1990). The model proposes that the progression from normal mucosa to cancer requires at least five genetic alterations, commonly found in *APC*, *p53* and *K-RAS* as well as in genes mapped on the long arm of chromosome 18. Understanding of molecular pathways leading to CRC has advanced remarkably since the original adenoma-carcinoma sequence model was proposed. Now, almost three decades later, it is recognized that instead of relatively limited repertoire of genetic alterations proposed in the original model, there are several genetic and epigenetic alterations that drive CRC formation through multiple molecular pathways (Grady & Lao, 2011). Although a large number of genes have been associated with colorectal cancer, only few have been found to be altered in sizeable proportion. As Fearon and Vogelstein proposed, genes involved in Wnt/β-catenin, Ras, and p53 signalling still seem to be the most widely altered genes in CRC tumors (Schell et al., 2016).

Alterations in the tumor suppressor gene *APC* is found in up to 80% of sporadic colorectal carcinomas and it is thought to be the initial mutation in most of the colorectal adenomas (Humphries & Wright, 2008). APC is involved in the Wnt-signaling, which has a central role in the intestinal epithelial renewal (Fevr, Robine, Louvard, & Huelsken, 2007). APC is suggested to play a role in the regulation of crypt fission, and in fact, elevated crypt fission rates have been reported in mice with germline

mutation in *APC* (Wasan et al., 1998). The main tumor suppressor function of APC is likely based on its ability to regulate the intracellular β-catenin levels by taking part in the formation of a protein complex which eventually leads to cytoplasmic β-catenin degradation in the absence of Wnt-signaling (Smits et al., 1999). Wnt-signal prevent the degradation of β-catenin, which leads to β-catenin translocation into the nucleus where it activates transcription of Wnt-responsive genes (MacDonald, Tamai, & He, 2009). Alterations in *APC* gene might disrupt the function of APC protein, thus preventing the degradation of β-catenin, even in the absence of Wnt-signal, resulting in uncontrolled transcriptional activation of oncogenes, which in turn might contribute to the cancer development (Korinek et al., 1997).

K-RAS oncogene is mutated in 30-50% of human CRCs (Pino & Chung, 2010). *K-RAS* codes a membrane-anchored GTPase, KRAS, which transmits intracellular signals when GTP is bound. Wild-type KRAS is inactivated by GTP hydrolysis. Majority of *K-RAS* mutations associated with CRC prevent the GTP hydrolysis, therefore causing KRAS to remain in the active form and activate pathways involved in cell proliferation and differentiation (Tan & Du, 2012). Activating *K-RAS* mutations are also suggested to drive the CRC by accelerating the crypt fission rates (Snippert, Schepers, van Es, Simons, & Clevers, 2014). The primary downstream target of KRAS is a serine-threonine protein kinase BRAF, which is mutated in approximately 10% of CRCs (Barras, 2015). Interestingly, *K-RAS* and *BRAF* mutations never occur in the same cell (Pegah Larki et al., 2017). Cells harbouring both mutations are prone to senescence, which probably confers negative selection and is the reason for mutually the exclusive nature of *K-RAS/BRAF* mutations (Cisowski, Sayin, Liu, Karlsson, & Bergo, 2016).

Tumor suppressor p53 is a transcription factor that activates a number of genes involved in cell cycle arrest, senescence, and apoptosis (X. Li, Zhou, Chen, & Chng, 2015). Mutations leading to loss of function or abnormal function of p53 are associated with several types of cancers (Kandoth et al., 2013). Majority of *TP53* mutations associated with CRC alters the DNA binding domain which leads to the synthesis of a partially inactive protein (X. Li et al., 2015; Russo et al., 2005).

The long arm of chromosome 18 include several tumor suppressor genes, and loss of heterozygosity (LOH) in this particular region is detected in up to 70% of CRCs (Armaghany, Wilson, Chu, & Mills, 2012). For instance, the genes coding transcription factors SMAD2 and SMAD4 locate in 18q and are frequently mutated in CRC (Fleming et al., 2013). SMAD2 and SMAD4 are involved in the

transforming growth factor beta (TGF-β) signaling pathway, which functions to suppress cellular proliferation, differentiation and apoptosis (Weidong Xu et al., 2000).

2.2 Genomic instability in CRC

Genomic instability is a hallmark of cancer (Hanahan & Weinberg, 2011). In the CRC, loss of genomic stability is thought to occur early in cancer development and drive malignant transformation of cells by accelerating the accumulation of genetic alterations in oncogenes and tumor suppressor genes (Pino & Chung, 2010b). CRC pathways are classified into three subtypes based on the type of genomic instability: chromosomal instability (CIN), microsatellite instability (MSI), and the CpG island methylator phenotype (CIMP) (Kuipers et al., 2015). CIN, the most common CRC pathway, is characterized by a high rate of chromosomal alterations and widespread LOH (Lengauer, Kinzler, & Vogelstein, 1997). The MSI pathway is a consequence of the defective mismatch repair (MMR) mechanism. CIMP is characterized by global genome hypermethylation resulting in tumor suppressor gene inactivation (Nazemalhosseini Mojarad, Kuppen, Aghdaei, & Zali, 2013). It should be noted that these pathways are not mutually exclusive, and some tumors are reported to exhibit features from more than one pathway (Goel et al., 2003). Also, some mutations, such as those in *APC*, are common among all the groups suggesting an important role in colorectal cancer in general (Kuipers et al., 2015).

2.2.1 Chromosomal instability

The vast majority (65-70%) of colorectal cancers display high rate of structural and numerical chromosome alterations, a phenomenon termed chromosomal instability (CIN) (Lengauer et al., 1997; Pino & Chung, 2010). Besides large chromosomal alterations, CIN tumors often exhibit widespread LOH in the tumor suppressor gene loci. Mutation in *APC*, *K-RAS* and *TP53*, the most commonly observed alterations in CRC, are frequently found in CIN tumors (Armaghany et al., 2012). However, it is not clear if CIN accelerates the acquisition of these mutations or vice versa (Pino & Chung, 2010).

Chromosome segregation is a critical step in cell division, where sister chromatids are pulled towards opposite poles, thereby ensuring that each daughter cell receives normal chromosome content. Spindle assembly checkpoint (SAC) maintains genomic stability by delaying the methaphase-to-

anaphase transition until all chromosomes are properly aligned (Lara-Gonzalez, Westhorpe, & Taylor, 2012). Defective SAC may cause CIN by increasing segregation errors. *Budding uninhibited by benzimidazoles 1 (BUB1)* is essential for SAC assembly, as it is required for the recruitment of other SAC proteins (V. L. Johnson, Scott, Holt, Hussein, & Taylor, 2004). Mutations in *BUB1* have been associated with CRC (Jaffrey et al., 2000).

The mitotic spindle is responsible for pulling chromosomes towards the opposite poles. Aurora-A kinase, encoded by the *AURKA* gene, is essential for spindle assembly. The interaction between Aurora A and the spindle microtubules is mediated by a microtubule-associated protein TPX2 (Thomas A. Kufer et al., 2002). Recent findings indicate that alterations in *TPX2* contributes to invasion and metastasis of colon cancer (Ping Wei et al., 2013). Furthermore, it was recently demonstrated that overexpression of AURKA and loss of Chk2, features commonly found in colorectal carcinomas, promote CIN by increasing microtubule assembly rates in colorectal cancer cells (Ertych et al., 2014). Increased microtubule assembly rates supposedly lead to hyperstable microtubule-kinetochore attachments, thereby increasing segregation errors (Ertych et al., 2014).

CRC tumors characterized by CIN frequently contain both numerical and structural chromosomal aberrations. An abnormal chromosome structure is often caused by defective DNA replication and damage response, and in fact, genes associated with these functions have been reported to be misregulated in CRCs (Pillaire et al., 2010). For instance, RAD9 Checkpoint Clamp Component A (RAD9A) has an important role in DNA damage response and genomic stability maintenance, and its aberrant expression is associated with multiple cancers (Lieberman et al., 2011). RAD9A contributes to the cell cycle checkpoint control and multiple types of DNA repair: homologous recombination repair, base excision repair, mismatch repair and nucleotide excision repair (T. Li et al., 2013). RAD9A physically interacts with the MLH1 protein, which is a crucial component of the mismatch repair (MMR) system (He et al., 2008). Altered *MLH1* expression is strongly associated with microsatellite instability, however, a recent study reported a possible association between Mlh1 deficiency and CIN colon cancer (Pussila et al. 2018). MMR is known to suppress illegitimate mitotic recombination between sequences that share no homology (León-Ortiz et al., 2018). Recombination between non-identical sequences is a source of chromosome rearrangements, which poses a potential link between the MMR gene deficiency and CIN. The MMR system is further discussed in the "Microsatellite instability" section.

Centromeres and telomeres are chromosome structures composed of specific DNA sequences and associated proteins. The specialised structure of these chromosomal regions makes them especially prone to replication errors, which may lead to structural aberrations (Mankouri, Huttner, & Hickson, 2013). Telomeres are repetitive DNA sequences at the ends of chromosomes, where they protect chromosome ends from being recognized as DNA breaks. Abnormal telomere function might result in telomere fusion, thus generating acentric and dicentric chromosomes, which are frequently found in cancer cells (Aina Bernal & Laura Tusell, 2018; Ganem, Godinho, & Pellman, 2009). Centromeres act as attachment points for microtubules, and therefore, they are important for proper chromosome segregation. Centromere protein A (CENP-A) is essential for centromere function, and it must be carefully deposited at every newly replicated centromere. MIS18 Kinetochore Protein A (MIS18A) forms a heterodimer with MIS18B, which is required for CENP-A deposition (Fujita et al., 2007; Nardi, Zasadzińska, Stellfox, Knippler, & Foltz, 2016). *Mis18a* knockout causes severe defects in chromosomal segregation due to mislocalization of CENPA (Kim et al., 2012).

Several mechanisms have been suggested to contribute to CIN, yet the accurate causes and consequences of the pathway remain poorly understood. The process is likely to be heterogenous, including multiple chromosome segregation associated factors, such as those discussed above. This study focuses on six chromosome segregation associated genes, which are summarized in Table 2.

Table 2. Summary of the genes studied in this Master's thesis.

Gene	Function	Reference
BUB1	Codes Bub1, which is required for spindle-assembly checkpoint signalling and correct chromosome alignment	(V. L. Johnson et al., 2004)
MIS18A	Codes Mis18a, which is required for correct localization of CENP-A. Knockout causes defects in segregation.	(Kim et al., 2012)
RAD9A	Codes Rad9a, which has a major role in DNA damage response. Prevents non-homologous end joining.	(Li et al., 2013)
TPX2	Codes Tpx2, which is required for mitotic spindle assembly and function.	(Thomas A. Kufer et al., 2002)
MLH1	Codes Mlh1, which is a component of MMR system.	(Jascur & Boland, 2006)
PMS2	Codes Pms2, which is a component of MMR system.	(Jascur & Boland, 2006)

2.2.2 Microsatellite instability

A defective MMR system is the underlying cause for the microsatellite instability (MSI), which accounts for approximately 15% of all CRCs (Colussi, Brandi, Bazzoli, & Ricciardiello, 2013). Expanded or contracted microsatellite regions are a hallmark of a defective MMR system (Kuipers et al., 2015). The microsatellite size differences are caused by a replication error termed replication slippage, which is a consequence of temporary dissociation of the polymerase and template strand during replication (Viguera, Canceill, & Ehrlich, 2001). Since microsatellites are composed of repetitive sequences, the polymerase may not recognize the dissociation point in template strand, and therefore, it might reassociate in one or two repeats ahead or behind the accurate place. Normally, DNA MMR lowers the mutation rate by correcting mismatching nucleotides arising from replication errors, recombination and base modifications (G. Li, 2008). However, alterations in genes that contribute to the MMR mechanism might lead to defective correction of replication errors and accumulation of mutations, which is indicated by microsatellite instability (Lynch et al., 2009).

Approximately 20% of cancers showing MSI represent a hereditary colorectal cancer syndrome, Lynch syndrome (LS), which is caused by a germline mutation in one of the MMR genes (Colussi et al., 2013). Yet most of the MSI CRCs arise sporadically and acquire the MSI through epigenetic silencing of the *MLH1* promoter (Parsons, Buchanan, Thompson, Young, & Spurdle, 2012). The loss of the Pms2 protein and an activating mutation in *BRAF* are common features of sporadic MSI tumors (Boland & Goel, 2010). A specific mutation, $BRAF^{V600E}$, occurs in over 40% of MSI CRCs (Carethers & Jung, 2015). Furthermore, frequently mutated tumor suppressor genes *TP53* and *APC*, are significantly less commonly mutated in MSI tumors compared to CIN tumors (Carethers & Jung, 2015).

2.2.2 CpG island methylator phenotype

The third CRC pathway, CpG island methylator phenotype (CIMP), is observed in approximately 20% of CRCs (Nazemalhosseini Mojarad et al., 2013). DNA methylation, a covalent addition of methyl groups to DNA bases, is an epigenetic mechanism involved in several normal and essential mammalian functions, such as development, differentiation, transcriptional regulation, genomic stability maintenance and suppression of the activity of repetitive elements (Smith & Meissner, 2013). Aberrant methylation patterns, such as global hypomethylation and hypermethylation, have been found in many types of cancers (Ehrlich, 2002). The CIMP phenotype in CRC is characterized by

hypermethylation of CG rich areas in promoter regions, termed CpG islands, resulting in transcriptional silencing of several tumor suppressor genes (Al-Sohaily, Biankin, Leong, Kohonen-Corish, & Warusavitarne, 2012).

Although hypermethylation is a common feature of CIMP tumors, the pathway lacks a universally accepted defining criteria. Also, the molecular basis of CIMP development remain mainly unknown. Several methylation associated factors, such as overexpression of DNA methyltransferases and mutations in genes that contribute to chromatin remodelling, are suggested to underlie CIMP pathway, but none of those are yet shown to be consistent for CIMP CRCs (Carethers & Jung, 2015). CIMP tumors mainly arise from serrated polyps and display similar molecular features to sporadic MSI tumors, such as *BRAF* mutations, wild type *TP53* and MSI caused by *MLH1* promoter methylation (Carethers & Jung, 2015; Colussi et al., 2013).

CIMP tumors with high levels of methylated CpG sites are associated with old age (Ang et al., 2010). Interestingly, aberrant methylation is also seen in normal colon epithelial cells as a result of aging. These age-related changes in methylation patterns may predispose the mucosa to cancer formation by affecting genes that regulate the growth and/or differentiation of colon cells. Age-related hypermethylation associated with colorectal cancer is termed type-A methylation. The type-C methylation, by contrast, is exclusively seen in cancer, and is associated with the CIMP phenotype. (Toyota & Issa, 1999).

2.3 Field defect in colon

The first genetic alterations towards CRC occur in normal appearing colorectal epithelia (Luo, Yu, & Grady, 2014). The clonal expansion of cells harbouring these cancer predisposing alterations can lead to the formation of fields of tissue that consist of cells possessing some but not all features required for malignancy (Curtius et al., 2018). This process is termed field defect, or field cancerization, first introduced by Slaughter et al. (1953) when studying oral cancer, but have since been described also in other cancer types (Bernstein, Bernstein, Payne, Dvorak, & Garewal, 2008). The first suggestions of field defect in colon arouse from the increased occurrence of flat dysplasia and CRC in patients with inflammatory bowel disease (Luo et al., 2014). Tumors associated with chronic inflammation typically arise from flat mucosa that has become dysplastic (Luo et al., 2014). The same genetic alterations found in tumor tissue are shown to exist in the normal appearing mucosa next to the tumor,

providing evidence of field defect in chronic inflammation associated CRC (Galandiuk et al., 2012). In sporadic CRC, the suggestions of field defect originated from the observation that patients with personal history of CRC have exceedingly high incidence of second colorectal tumor, as compared to the general population (Levi et al., 2013; Luo et al., 2014). Furthermore, studies of tumor adjacent normal colon mucosa have recognized cells with aberrant karyotypes, epigenetic profiles and gene expression patterns, suggesting the existence of pre-cancerous field (Hawthorn, Lan, & Mojica, 2014; Pussila et al., 2018; Shen et al., 2005).

Despite the large body of supportive evidence, the mechanisms and consequences of field defect in colon remain poorly understood. Clonal expansion seems to be the most widely discussed mechanism contributing to the formation of pre-cancerous field in colon. In this model, the initial alteration towards cancer enables the mutant cell to outcompete other crypt cells, which leads to the replacement of all crypt cells by mutant cells (Humphries & Wright, 2008). The cells acquire more mutations during the proliferation, which further promote clonal expansion, for example by accelerating crypt fission (Curtius et al., 2018). Crypt fission generates a patch of mucosa with crypts filled with mutated cells and, eventually, some of these cells gain features that are enough for malignant phenotype and tumor formation (Fig. 3a) (Curtius et al., 2018). If carcinoma arises through clonal expansion, the cells would have derived from single clone, and therefore, the adenoma would be monoclonal. However, some colorectal adenomas are reported to be polyclonal, which means that they have originated from multiple independent clones instead of just one (Richard B. Halberg & William F. Dove, 2007). One possible explanation for polyclonality is the independent field formation by multiple cell lineages (Fig. 3b). For instance, carcinogenetic diet might promote mutations or epigenetic alterations in colon mucosa, causing some cells to gain advantageous mutations (Luo et al., 2014). This in turn could lead to clonal expansion of mutant cells and eventually to the formation of a polyclonal field which is composed of several pre-malignant cell lineages (Curtius et al., 2018). It has also been suggested that the appearance of abnormal mucosa found next to the tumor would not be a primary event, instead the field is proposed to be a secondary effect caused by the presence of the tumor. In this scenario, the clonal population of initial mutant cells would have some kind of impact to the adjacent crypt cells, for example by aberrant signalling, thus resulting in field defect (Fig. 3c) (Patel, Tripathi, Gopalakrishnan, Williams, & Arasaradnam, 2015).

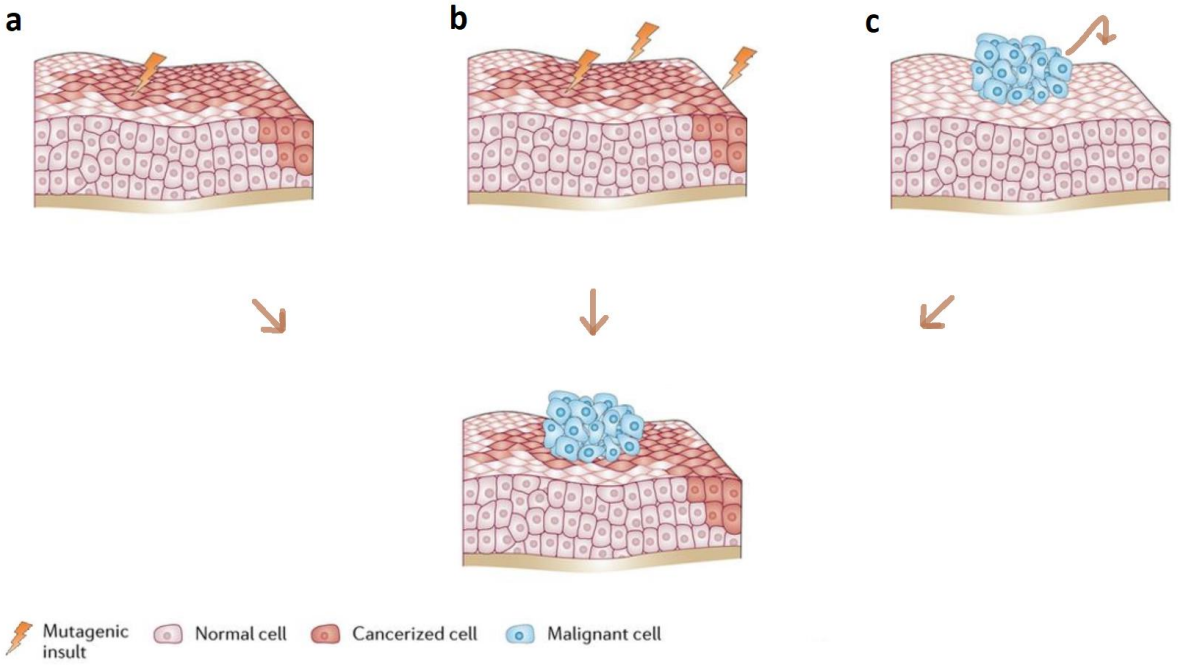

Fig. 4. Origin of an abnormal field surrounding the tumor. A. Mutant cell lineage expand in the tissue, thus forming a pre-cancerous field in which the tumor is more likely to arise. B. Several cell lineages gain malignant features independently, expand in the tissue and give rise to a polyclonal field in which the tumor is more likely to arise. C. Field defect as a secondary effect: established tumor affects the adjacent cells, which leads to the formation of a tumor surrounding field of abnormal cells. Modified from Curtius et al. (2018).

Characterization of the molecular events that occur in the pre-cancerous field could enable the identification of the earliest steps in CRC formation (Patel et al., 2015). From the diagnostic point of view, determination of a set of molecular features that reliably predict the increased risk of developing malignancy is highly desirable. Individuals at high risk could be selected for screening programs thereby ensuring the early detection of tumors and prevention of cancer development. Another exciting prospect of the field defect concept is the possibility of preventing the cancer development by chemopreventive therapies. Targeted therapy could, for instance, reduce the mutation rate and change the microenvironment such that certain clones that are already established in the pre-malignant field would no longer be selected for, thus arresting the malignant transformation (Curtius et al., 2018). Besides the diagnostic advantages, determination of the pre-cancerous field could help optimizing the surgical margins when removing the tumor and, therefore, potentially lower the risk for second primary tumor.

2.4 Mouse models of CRC

Environmental factors and genetic heterogeneity among humans add complexity to the molecular etiology, pathology, and clinical progression of cancer, which make it challenging to study the disease mechanisms. However, the sources of variation caused by genetic background and environmental factors can be minimized by using inbred animal strains and controlling the experiment conditions. Due to the small size, physiological similarity to human and the ease of breeding them in the laboratory, mice have been long used as models for human development and disease (Perlman, 2016).

Mouse intestine, especially the colonic crypt structure, highly resembles that of the human. However, there are few major anatomical differences (Fig. 5). For instance, human have a proportionately smaller cecum when compared to mouse. Also, human colon is clearly divided into three sections (ascending, transverse and descending colon) and has a segmented appearance caused by small pouches called haustra, in contrast to mouse colon, which is relatively smooth and lacks the division into three sections (Nguyen, Vieira-Silva, Liston, & Raes, 2015). In addition to colon anatomy, there are some differences in the colonic cell distribution (Nguyen et al., 2015). It is not clear whether the differences between mouse and human colon affect tumorigenesis, but it is important to take that possibility into consideration when making direct parallels between mouse and human CRC (Nguyen et al., 2015).

The first mouse model for human CRC was described in 1990 and named *Apc*^{Min} due to the mutation in *Apc* gene which resulted in multiple intestinal neoplasia (Min). Since the *Apc*^{Min} was described, several mouse models with *Apc* mutations have been constructed and used to study the development, treatment and prevention of human CRC containing *APC* mutations. Interestingly, unlike in human FAP, which is caused by germline *APC* mutations and characterized by multiple adenomas in colon, in *Apc* mutant mice the majority of intestinal adenomas develop into small intestine (R. L. Johnson & Fleet, 2013).

Several MMR deficient knockout mouse strains have been created to demonstrate the role of MMR in cancer development. Most of the MMR gene knockout mice are cancer prone and MSI-positive, highlighting the crucial role of MMR system (G. Li, 2008). However, instead of developing colon cancer, the MMR gene knockout mice often develop severe lymphoma and die at an early age (G. Li, 2008). Mutations in *MLH1* and *MSH2* are the most common causes of Lynch syndrome (Lynch et al., 2009). Homozygous MLH1 and MSH2 mice are MMR deficient and develop MSI tumors to

gastrointestinal tract, however, they locate more often in the small intestine than in the colon (Edelmann et al., 1996). Mice with one null *Mlh1* or *Msh2* allele (which is analogous to human LS) are MMR proficient (Boland, 2010). $Msh2^{+/-}$ mice do not have excess of early onset tumors like LS humans and $Mlh1^{+/-}$ mice (Boland, 2010). $Mlh1^{+/-}$ mice develop tumors with MSI, suggesting that the loss of MMR activity is acquired by somatic alterations and is probably a prerequisite to cancer development in $Mlh1^{+/-}$ (Edelmann et al., 1999). It has been argued that Mlh1 deficiency may drive the development of cancer even if the MMR system is still proficient. Indeed, a recent study showed that decreased level of *Mlh1* expression was associated to microsatellite stable CRC, suggesting an alternative link between *Mlh1* deficiency and CRC (Pussila et al., 2018).

Genetic modification has enabled the researchers to study how certain mutations contribute to cancer development. However, most of the human cancers arise sporadically without cancer predisposing germline mutations. When considering sporadic cancer, a general weakness of mouse models is the low incidence of spontaneous colonic tumors and cancer cell invasion (R. L. Johnson & Fleet, 2013). Therefore, along with genetic engineering, chemical treatments are often used to increase tumor incidence. Yet mutagenic chemicals cannot target specific human cancer relative genes, instead they induce mutations all over the genome. Importantly, feeding certain diets, such as those modified to resemble human Western diet, have been successfully used to induce hyperplasia and tumorigenesis in mouse colon (R. L. Johnson & Fleet, 2013).

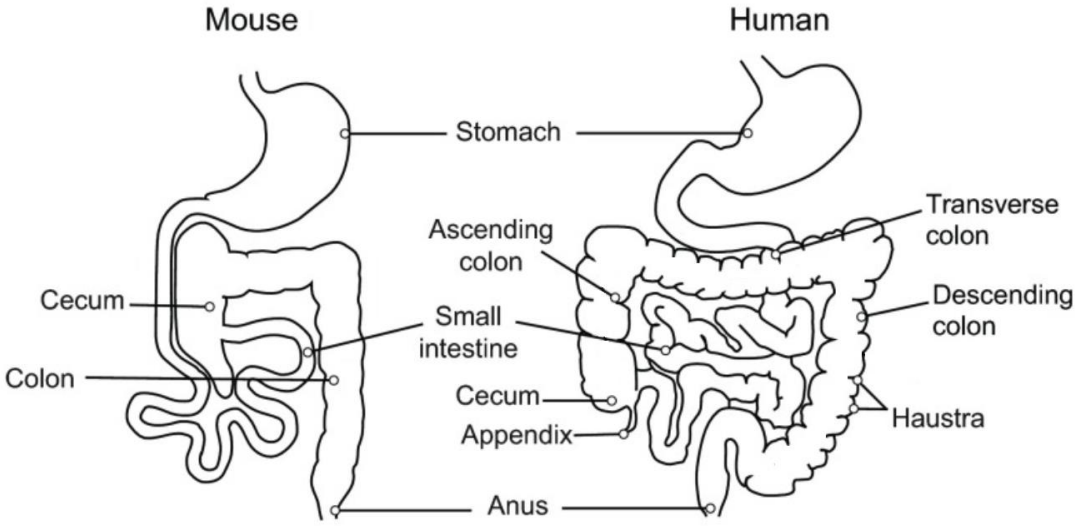

Fig. 5. Anatomy of the mouse and human gastrointestinal tract. Mouse cecum is proportionately larger than human cecum. The human colon is divided into different sections (i.e. ascending, transverse and descending colon) and compartmentalization in haustra, which are absent in the mouse colon. Modified from Nguyen et al. (2015).

3. AIMS OF THE STUDY

The aim of the Master's thesis was to revise and investigate whether the six chromosomal segregation genes, *Bub1, Mis18a, Pms2, Rad9a, Tpx2, and Mlh1*, would be aberrantly expressed in the histologically normal colon mucosa next to the carcinoma, and thereby, possibly signal the carcinogenesis in mouse colon. This was done by analysing the mRNA expression in the colon mucosa of carcinoma and non-carcinoma mice, and comparing the expression levels between the proximal colon, where all the carcinomas located, and the distal colon.

Specific aims:

1. To study mRNA expression of the genes *Bub1, Mis18a, Pms2, Rad9a, Tpx2,* and *Mlh1* in mouse colon mucosa.
2. To compare the gene expression profiles of the six genes in carcinoma and non-carcinoma mice.
3. To compare the gene expression profiles of the six genes in distal and proximal part of the colon.

4. MATERIALS AND METHODS

4.1 Mice

Heterozygote B6.129-Mlh1^{tm1Rak} (Mlh1$^{+/-}$) and wild-type C57BL/6 mice were obtained from NCI-MMHCC; National Institutes of Health, Mouse Repository, NCI-Frederick, MD. In heterozygote B6.129-Mlh1^{tm1Rak} mice, the exon 2 is replaced with neomycin cassette in one of the *Mlh1* alleles, resulting in absence of Mlh1 protein expression from the mutated allele (Edelmann et al., 1996). Six breeder pairs, formed by the *Mlh1*$^{+/-}$ mice and their wild-type C57BL/6 mates, produced the mice used in this study. The mice had been genotyped previously by Pussila et al. (2013). The mice were randomly divided into two dietary groups at the age of five weeks. One group was fed with healthy rodent control diet AIN93G (AIN) and the other group was fed with Western-style diet (WD) modified from AIN (Harlan Teklad, Madison, WI) (Pussila et al., 2013). The main difference between AIN and WD is the source of fat, since the majority (66,5%) of the fat in WD is animal (milk) fat, in contrast to AIN, where the fat source is soybean oil (Pussila et al., 2013). The mice were bred and treated according to study protocol approved by the National Animal Experiment Board in Finland (ESLH-2008-06502/Ym-23).

Twelve mice per each group (*Mlh1*$^{+/+}$ AIN, *Mlh1*$^{+/-}$ AIN, *Mlh1*$^{+/+}$ WD, *Mlh1*$^{+/-}$ WD), were sacrificed at time point (tp) 0 (5 weeks of age, *Mlh1*$^{+/-}$, *Mlh1*$^{+/+}$), tp1 (12 months of age), tp2 (18 months of age) and tp3 (21 months of age) (Pussila et al., 2018). Altogether 14 mice were selected to this study, of which one was 12 months of age and the rest 13 were 18 months of age at the time of sacrifice. Six of the mice (E249, E314, E329, E333, E338 and E347) had been found to have a proximal colon carcinoma. The other eight mice (E306, E311, E312, E320, E322, E325, E332 and E346) included in the study did not have a colon carcinoma, and therefore, served as control samples. All the mice included in the study, as well as their genotypes, ages, diets and their intestinal findings, are shown in Table 3.

Table 3. Characteristics of the mice used in this study.

ID	Genotype	Diet	Age	Sex	Intestinal finding
E249	wt	WD	12 mo	M	Tubular adenocarinoma
E314	wt	WD	18 mo	M	Mucinous adenocarinoma
E329	wt	AIN	18 mo	F	Tubulovillous adenocarinoma
E333	wt	WD	18 mo	F	Tubular adenocarinoma
E338	*Mlh1*$^{+/-}$	WD	18 mo	F	Tubular adenocarinoma
E347	*Mlh1*$^{+/-}$	WD	18 mo	F	Serrated adenocarinoma
E306	wt	AIN	18 mo	F	-
E311	wt	WD	18 mo	M	-
E312	*Mlh1*$^{+/-}$	WD	18 mo	M	-
E320	wt	AIN	18 mo	M	-
E322	wt	WD	18 mo	M	-
E325	wt	AIN	18 mo	M	-
E332	wt	AIN	18 mo	M	-
E346	*Mlh1*$^{+/-}$	WD	18 mo	F	-

4.2 RNA extraction and quality control

The distal and proximal colonic mucosa samples used in this study had been collected previously and stored in RNAlater (Qiagen, Hilden, Germany) in -80°C. RNA was extracted from mucosa samples using RNeasy Plus Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's protocol with an extra on-column DNase treatment using RNase free DNase set (Qiagen, Hilden, Germany) to prevent genomic DNA contamination, and Reagent Dx (Qiagen, Hilden, Germany) to prevent foaming during tissue disruption. Tissue was shredded with scalpel and further disrupted and homogenized with TissueLyser LT (Qiagen, Hilden, Germany) for 2 x 2 minutes, 50Hz, using 7mm stainless steel beads (Qiagen, Hilden, Germany). RNA was eluted to 40µl of RNase free water and the RNA samples were stored in -80 °C. RNA concentrations were measured using Qubit 3.0 Fluorometer (Invitrogen, USA) with Qubit™ RNA HS Assay Kit (Life Technologies, Eugene, OR, USA) following the manufacturer's instructions. RNA integrity was analysed with Agilent 2100 Bioanalyzer (Agilent technologies, Santa Clara, CA) according to manufacturer's instructions.

4.4 cDNA synthesis and analysis of synthesis efficiency

The complementary DNA (cDNA) synthesis was performed following the recommendations provided by the manufacturer of the RT-qPCR assay. Since the reference gene selection and the actual gene expression analysis was done with two different assays, also the cDNA synthesis was performed using two different kits, RT^2 First Strand Kit (Qiagen, Hilden, Germany) and SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, USA), according to manufacturer's protocols. 100 ng of extracted RNA was used as a starting material for each reaction. The success of cDNA synthesis was verified by S15 PCR-method, which is based on the amplification of a highly conserved region of constitutively expressed *ribosomal protein S15* gene. S15 RNA is present in every tissue, and therefore it should be amplified while performing S15 PCR with successfully synthesised cDNA. S15 primers (Table 4.) amplify a product of the same size from both S15 cDNA and genomic DNA (gDNA). To rule out possible gDNA contamination, S15 PCR was also done to the RNA samples. PCR was done with Eppendorf™ Mastercycler™ Nexus Thermal Cycler with the following parameters: initial denaturation at 94°C for 10 minutes, 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, followed by a final extension at 72°C for 5 minutes. The PCR products were run on a 1.5% agarose gel in 1x sodium borate buffer for 60 minutes with 120 volts.

Table 4. S15 primer sequences.

S15 reverse	5'-CGG GCC GGC CAT GCT TTA CG-3'
S15 forward	5'-TTC CGC AAG TTC ACC TAC C-3'

4.4 Reference gene selection

Suitable reference genes were selected from the panel of 12 housekeeping genes; *actin beta (Actb), beta-2-microglobulin (B2m), glyceraldehyde-3phosphate dehydrogenase (Gapdh), glucuronidase beta (GusB), heat shock protein 90 alpha family class B member 1 (Hsp90ab1), lactate dehydrogenase A (Ldha), phosphoglycerate kinase 1 (Pgk1), peptidylpropyl isomerase H (Ppih), succinate dehydrogenase complex flavoprotein subunit A (Sdha), TATA-box binding protein (Tbp),*

transferrin receptor (Tfrc) and *ubiquitin C (Ubc).* The ideal reference gene should be expressed at constant level thus enabling the normalisation of differences in the amount of cDNA between samples. The gene expression analysis for the housekeeping genes was done using the RT2 Profiler™ PCR Array Mouse Housekeeping Genes (Qiagen, Hilden, Germany) and RT^2 SYBR Green qPCR Master mix (Qiagen, Hilden, Germany) according to manufacturer's protocol. The cDNA synthesized with RT^2 First Strand Kit (Qiagen) was used as a template in RT-qPCR reaction, as recommended in the RT^2 Profiler PCR Array Protocol. RT-qPCR was done with Bio-Rad CFX384 using the following parameters: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. The fluorescence data was acquired during the 60°C step.

4.5 RT-qPCR

The expressions of six genes related to chromosome segregation; *Bub1, Mis18a, Mlh1, Pms2, Rad9a* and *Tpx2* were analysed with RT-qPCR. *Ppih, Sdha* and *Ldha*, were used as reference genes. The studied genes had been reported to show expression differences between carcinoma and non-carcinoma mice in a previous study, and therefore, were selected for further analysis (Pussila et al., 2018). The expression levels were measured with Bio-Rad's CFX384 using TaqMan chemistry, where a specific probe including a fluorescent dye FAM and a quencher is utilized to quantitate cDNA in a sample. Probe anneals to its specific target sequence, where it is cleaved by *Taq* DNA polymerase, thus separating the quencher from the FAM dye, resulting in an increase in the fluorescence intensity proportional to the amount of PCR product. RT-qPCR was performed according to manufacturer's protocol using TaqMan gene expression assays (20x) (Applied Biosystems, USA), and TaqMan universal master mix II, no UNG (Thermo Fisher Scientific, Waltham, USA). The ID numbers for each assay are shown in Table 5. As recommended in the TaqMan gene expression assay protocol, the cDNA used as a template was prepared using SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific), and each sample was loaded in triplicates to control technical variation, such as pipetting errors. Furthermore, a pooled cDNA from non-carcinoma mice samples was used as a calibrator to remove between-run variation. Altogether three RT-qPCR runs were required to analyse all the samples. Each run included all the six genes of interest and the three reference genes. Analysis of *Pms2* expression was repeated in additional (4.) run. This additional *Pms2* analysis was performed using the distal colon cDNA samples that were synthesized with SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) during the present study, and distal colon cDNA samples that were synthesised with RT^2 First Strand Kit (Qiagen) and used in the previous study

performed by Suvi Rantamo (2017). In all the RT-qPCR runs, the samples were randomly assigned to the plates and wells, however, so that triplicates were in neighbouring wells. Following parameters were used in RT-qPCR runs: 95°C for 10 min, 40 cycles of 95°C for 15 s, and 60°C for 1 min. Fluorescence data was acquired during the 60°C step. Reaction components and their quantities are shown in Table 6.

Table 5. TaqMan assays for studied genes.

Gene	Assay ID number
Mlh1	Mm00503449_m1
Pms2	Mm01200871_m1
Rad9a	Mm00487923_m1
Tpx2	Mm01245970_m1
Bub1	Mm00660135_m1
Mis18a	Mm01209645_m1
Ldha*	Mm01612132_g1
Sdha*	Mm01352366_m1
Ppih*	Mm03024075_m1

* reference gene

Table 6. RT-qPCR reaction components.

RT-qPCR reaction mix (1x)	
Reagent	Volume
TaqMan Mastermix 10x No UNG	5 µl
TaqMan Assay 20x	0.5 µl
cDNA	1 µl
RNA free water	3.5 µl
Total	10 µl

4.6 Statistical methods

Bio Rad CFX Manager 3.0 software was used to handle information derived from the CFX384 Touch Real-Time PCR Detection System. The quantification cycle (Cq) values, which are the numbers of PCR cycles in which a real signal from the amplifying target molecule is detected, were exported to Excel for further analysis. In order to determine suitable reference genes, the standard deviations of the Cq values were calculated for each candidate reference gene. Three reference genes with the lowest standard deviations were selected for data normalization. The relative gene expression changes of the six genes of interest were analysed using the $2^{-\Delta\Delta Cq}$ method with *Sdha, Ldha* and *Ppih* as reference genes and the pooled cDNA from control samples as calibrator. For details and derivation of $2^{-\Delta\Delta Cq}$ see Livak & Schmittgen (2001). Briefly, the normalization factor for each sample was defined by calculating the mean Cq-value of the reference genes, which was subtracted from the Cq-values of the tested genes to get the ΔCq-values. To get the ΔΔCt, the ΔCq values of the plate calibrator samples were subtracted from the ΔCq-values of the actual samples. Finally, the gene expression fold changes were worked out by calculating two to the power of negative ΔΔCt ($2^{-\Delta\Delta Cq}$). SPSS 24 (IBM Corp., Armonk, NY, USA) was used to construct graphs and test statistical significance with Wilcoxon rank sum test (significance level set at 0.05).

5. RESULTS

5.1 RNA quantitation and quality control

The RNA concentrations varied between 17 and 418 ng/µl (Table 7). This was likely caused by varying amounts of tissue available for RNA extraction. Proximal samples of E320, E325 and E332 had only minute amount of tissue, which explains the low concentrations. RNA Integrity Number (RIN), which is used to assess the RNA quality, was 8.5 or higher for all the samples, indicating that RNA was intact. The S15 PCR amplification with all the samples synthesized with VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific) showed a ~ 360bp band when ran on the agarose gel, indicating that the RNA was successfully converted to DNA in the cDNA synthesis (Fig. 6 A). The absence of amplified product after performing S15 PCR to the RNA samples and running the reactions on agarose gel confirmed the absence of gDNA contamination (Fig. 6 B). Eleven out of fourteen cDNA samples synthesised with the RT^2 First Strand Kit (Qiagen) showed the ~ 360bp band on the gel, and the cDNA synthesis was, therefore, considered successful (Fig. 6 C). However, three samples (E249 CPR, E249 CD and E338 CD) synthesised with RT^2 First Strand Kit did not show any band when ran on agarose gel, indicating that either the cDNA synthesis or S15 PCR was unsuccessful. Therefore, the S15 PCR and gel electrophoresis were performed again for respective samples, this time with a successful result (Fig. 6 D).

Table 7. RIN-values and concentrations of the RNA-samples.

Sample ID	Concentration (ng/µl)	RIN	Sample ID	Concentration (ng/µl)	RIN
E249 CPR	52	9.4	E249 CD	131	9.6
E306 CPR	105	9.8	E306 CD	193	9.8
E311 CPR	40	9.4	E311 CD	91	10
E312 CPR	132	9.8	E312 CD	300	9.6
E314 CPR	176	9.5	E314 CD	286	9.7
E320 CPR	23	9.3	E320 CD	378	9.7
E322 CPR	87	9.7	E322 CD	185	9.7
E325 CPR	17	8.5	E325 CD	170	10
E329 CPR	78	9.6	E329 CD	58	9.6
E332 CPR	29	9	E332 CD	112	9.9
E333 CPR	106	9.4	E333 CD	150	9.7
E338 CPR	178	9.4	E338 CD	418	8.9
E346 CPR	182	10	E346 CD	210	9.9
E347 CPR	101	9.2	E347 CD	412	9.5

CPR = proximal colon, CD = distal colon

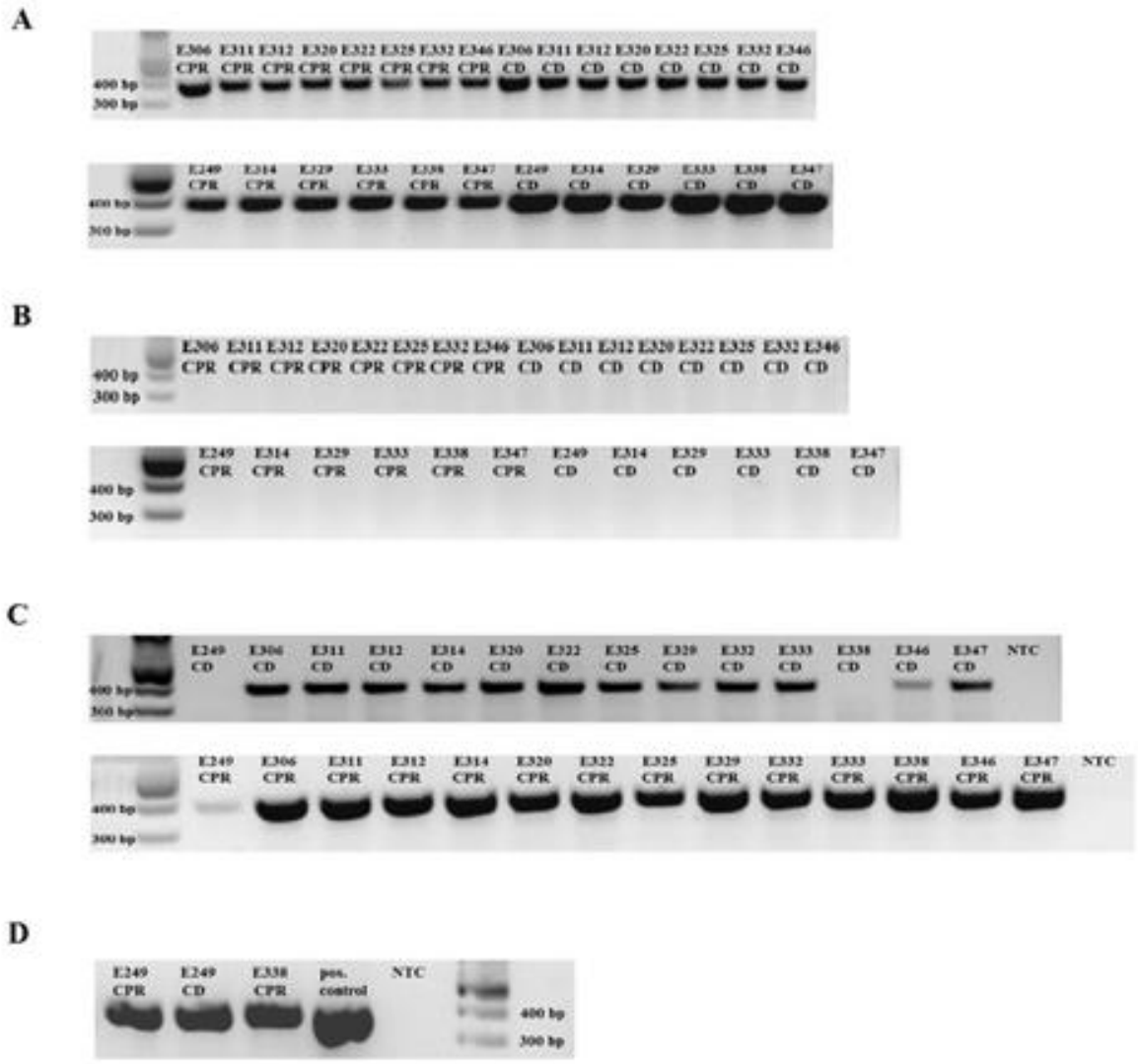

Fig. 6. S15 PCR products on agarose gel. A. Samples synthesized with VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific). All samples show the ~ 360bp band. B. RNA samples. Nothing has amplified in the S15 PCR, indicating the absence of gDNA contamination. C. Samples synthesized with RT^2 First Strand Kit (Qiagen). Eleven out of fourteen samples show the ~ 360bp band. D. The S15 PCR was per
ed again to the three samples synthesized with RT^2 First Strand Kit (Qiagen). All samples show the ~ 360bp product.

5.2 Reference genes

Stably expressed reference genes were selected based on the standard deviations (SD) of candidate reference gene Cq-values (Table 8). *Ldha*, *Ppih* and *Sdha* showed the lowest SD values and were, therefore, selected as reference genes for the following gene expression analysis. The mean Cq values

of the 12 candidate genes varied between 25,02 and 32,85, and the standard deviations variated between 0,71 and 1,21.

Table 8. Standard deviations (SD) and mean values of each candidate reference genes quantitation cycles (Cq).

Gene	SD	Mean Cq
Actb	0,82	25,02
B2m	0,80	26,67
Gapdh	0,86	27,01
Gusb	0,80	32,03
Hsp90ab2	0,85	27,43
Ldha	0,71	26,78
Pgk1	0,90	28,33
Ppih	0,78	31,81
Sdha	0,80	28,81
Tbp	0,96	32,85
Tfrc	1,21	30,83
Ubc	0,83	26,78

5.3 Effect of carcinoma status on expression profiles

The general expression levels for the colon were determined by combining the proximal and distal expression measurements and calculating the mean value for those. These were then compared between carcinoma and control mice in order to investigate whether the carcinoma status affected the overall gene expression levels in the colon (Fig. 9). The expression profile was somewhat equal between the two groups. Although the expression of *Bub1* and *Mlh1* seemed slightly lower, and *Rad9a* slightly higher in carcinoma mice, the differences were not statistically significant. The effect of carcinoma status was also investigated separately in the proximal colon, where all the carcinomas developed, and in the distal colon. Both the proximal (Fig. 10) and distal (Fig. 11) colon showed highly similar expression profiles in the carcinoma and control mice. Interestingly, in the distal colon, control mice showed higher *Mlh1* expression than carcinoma mice (p=0,046) (Fig.11).

In previous study, *Pms2* was reported to be significantly upregulated in the distal colon of the carcinoma mice when compared to the control mice (Rantamo, 2017). Here, the *Pms2* expression did not show any statistically significant differences between carcinoma and control mice. In order to clarify the cause for the distinct results, the same cDNA samples used in the previous study were re-analysed. The observed Cq values were higher for the samples prepared in the previous study than

for the samples prepared in the present study, which was likely caused by different cDNA synthesis efficiency. However, the observed relations in *Pms2* expression levels between carcinoma and control mice were highly similar in both sample sets (Fig. 12). Furthermore, the distal colon *Pms2* expression results from the repeated analysis were perfectly in line with expression levels observed in the first experiment in this study, indicating that most likely the distal colon *Pms2* expression was not affected by the proximal colon carcinoma.

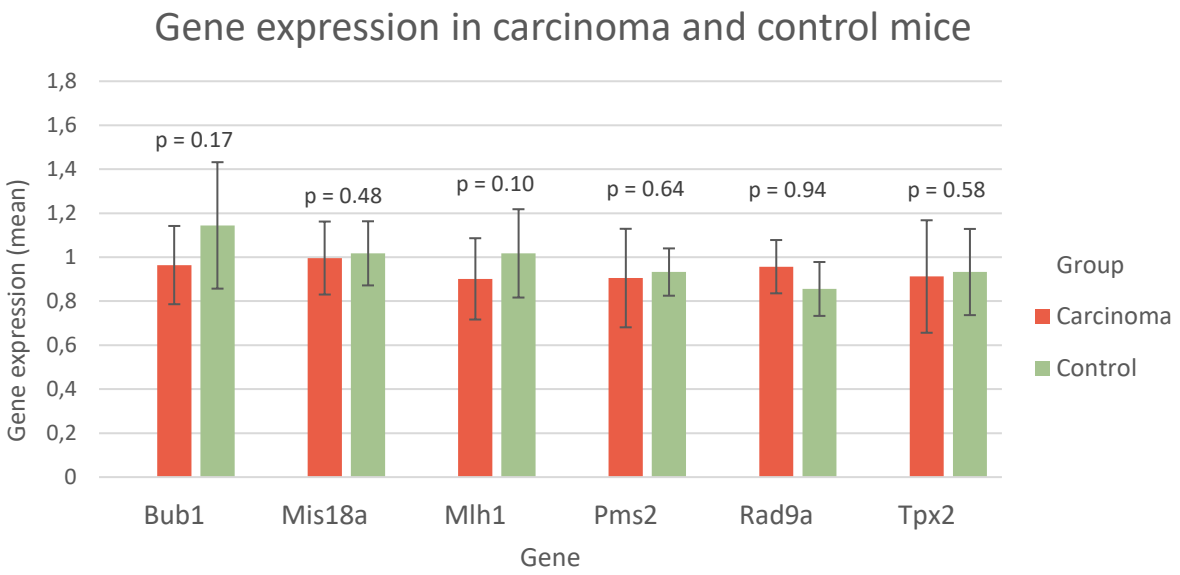

Fig. 9. The overall mean expression levels of carcinoma (n=6) and control (n=8) mice. The overall mean expression was calculated combining the proximal and distal colon expression measurements. The carcinoma status does not seem to affect the expression of the six studied genes in these mice. Error bars represent the 95% confidence intervals.

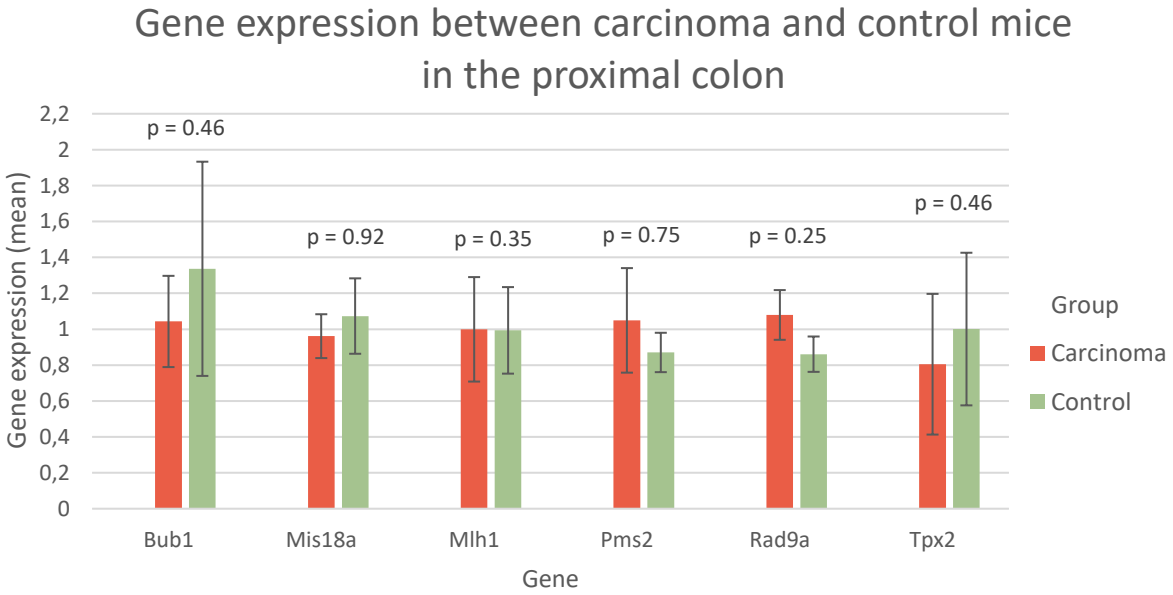

Fig. 10. The mean expression levels of carcinoma (n=6) and control (n=8) mice in the proximal colon. There are no statistically significant expression differences. Error bars represent the 95% confidence intervals.

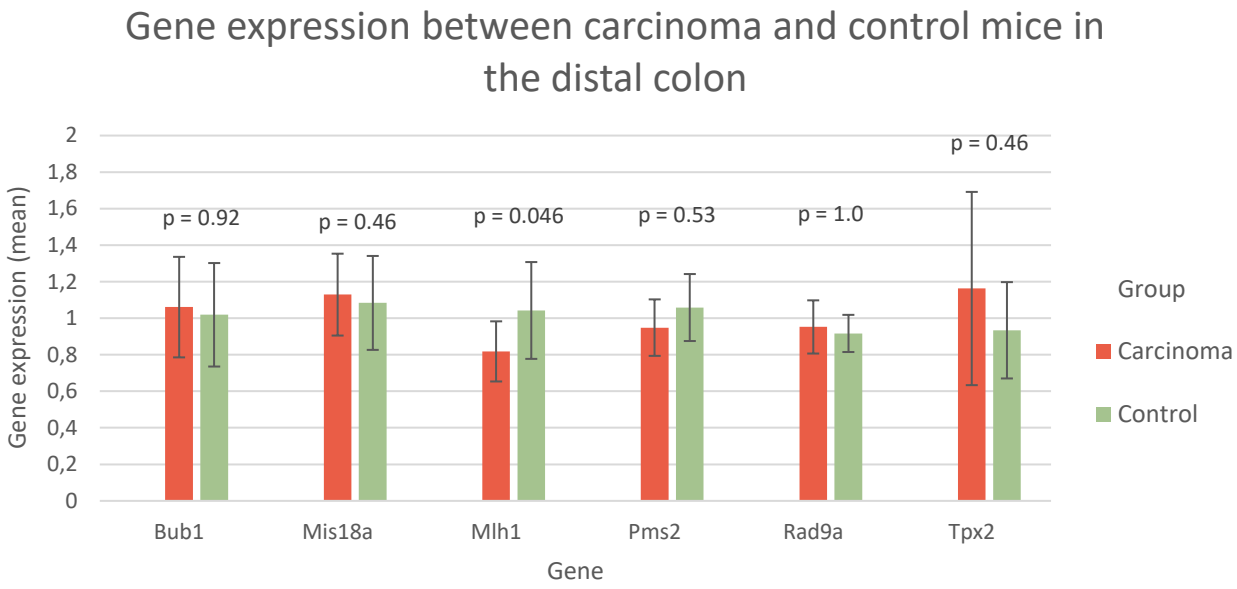

Fig. 11. The mean expression levels of the carcinoma (n=6) and control (n=8) mice in the distal colon. The expression is somewhat similar between carcinoma and control group, except for *Mlh1*, which is higher expressed in control mice (p=0,046). Error bars represent the 95% confidence intervals.

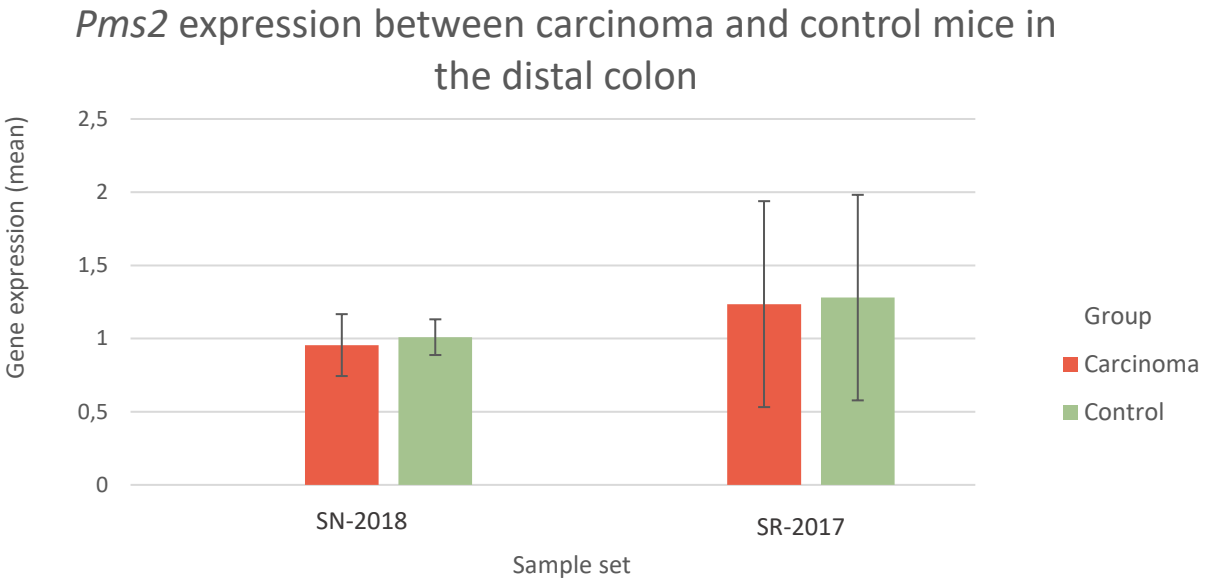

Fig. 12. Mean *Pms2* expression in samples used in this study (SN-2018) and samples used in the previous study (SR-2017). The relative gene expression between carcinoma (n=6) and control (n=8) mice was highly similar in both sample sets. Cq-values were higher in SR-2017 samples. Error bars represent the 95% confidence intervals.

5.4 Gene expression in distal and proximal colon

Mean expression levels of control mice's distal and proximal samples were compared to investigate whether there were normal regional expression differences in mouse colon (Fig. 13). Interindividual variation of expression levels within colon parts was wide, indicated by the long error bars. Again, the expression levels were observed to be somewhat equal, except for *Bub1*, which seemed to be expressed slightly more in proximal colon, and *Pms2*, which in turn seemed to be expressed slightly more in the distal colon. However, the differences were not clear enough reach statistical significance.

Regional expression levels were compared in carcinoma mice to study whether the carcinoma status affects the regional expression patterns in colon (Fig. 14). The expression profiles between proximal and distal colon samples were slightly different in control and carcinoma group. *Mis18a*, *Mlh1*, *Rad9a* and *Tpx2* showed larger local differences in carcinoma mice, although statistically not significant. Interestingly, in the carcinoma mice, the expression of *Bub1* was observed to be almost equal in the distal and proximal colon, indicating that the possible regional difference in *Bub1* expression observed in the control mice was not present in the carcinoma mice. This is illustrated more clearly in Fig. 15.

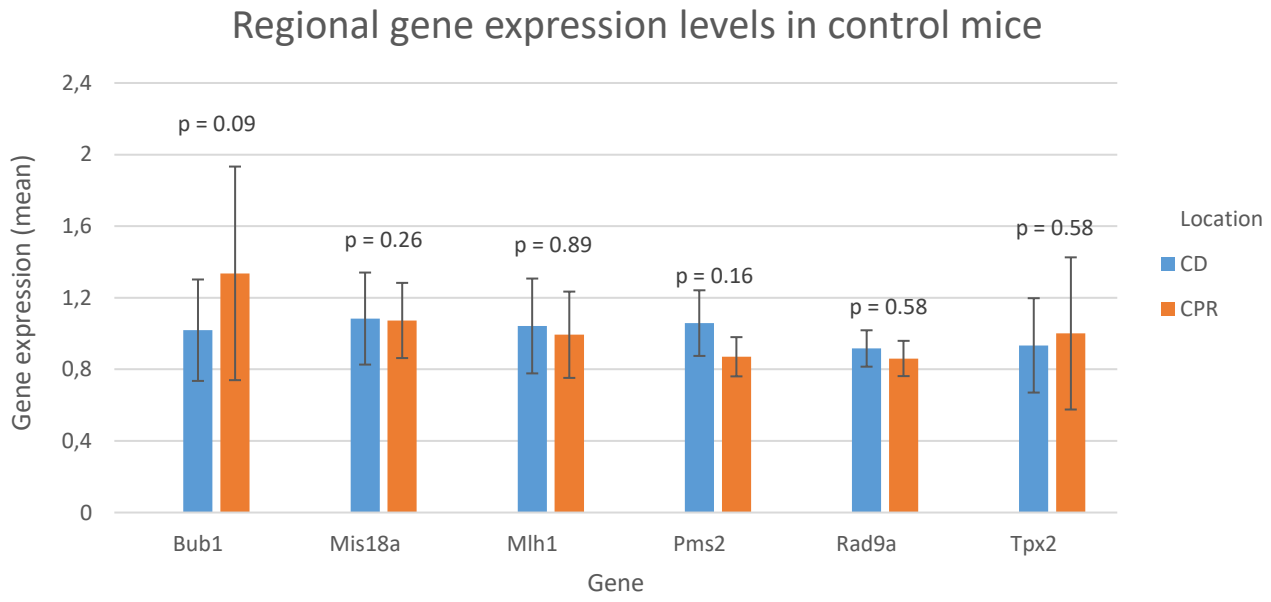

Fig. 13. Regional mean expression levels in the control mice. None of the six studied genes show statistically significant expression difference between the proximal (CPR) and distal (CD) colon in the control mice (n=8). Error bars represent the 95% confidence intervals.

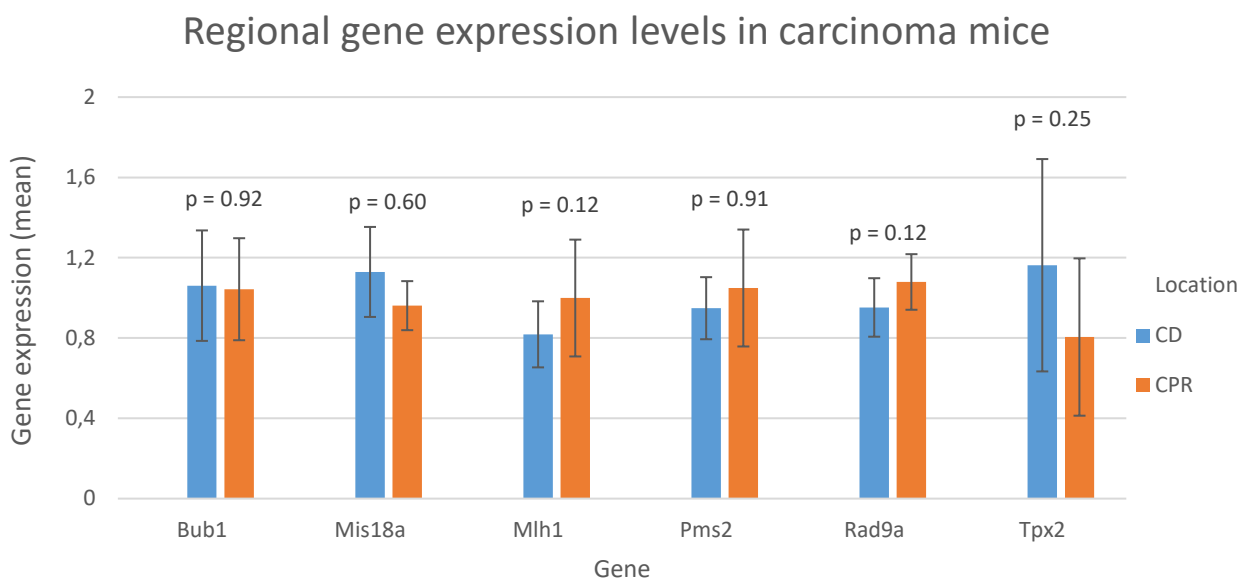

Fig. 14. Local mean expression levels of carcinoma mice. None of the six studied genes show statistically significant expression difference between the proximal (CPR) and distal (CD) colon in the carcinoma mice (n=6). Error bars represent the 95% confidence intervals.

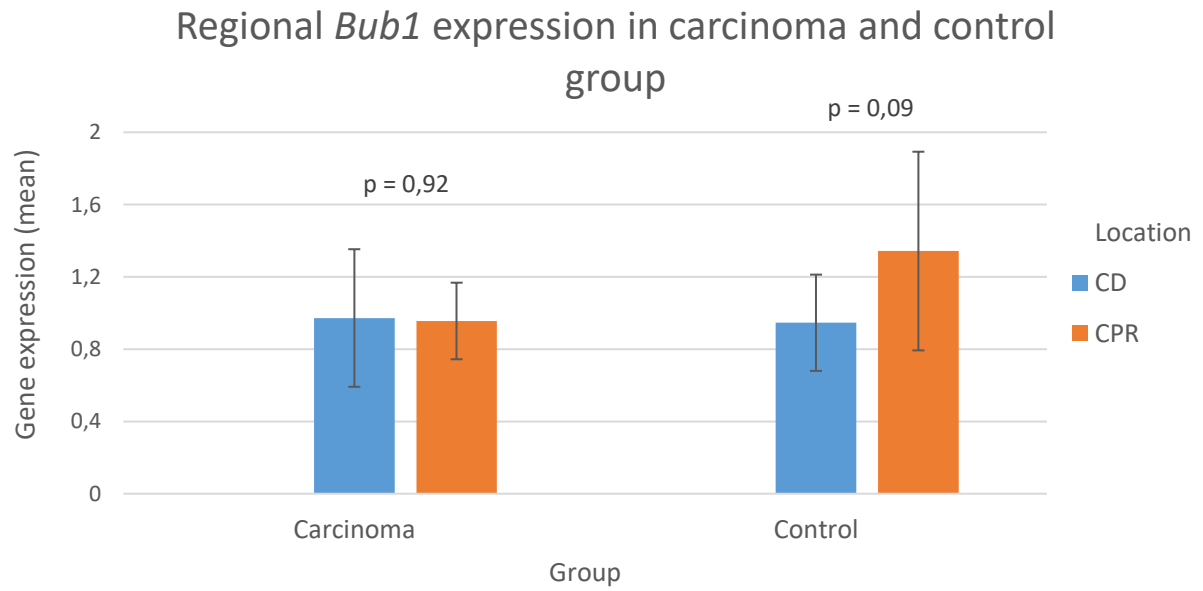

Fig. 15. *Bub1* local expression in carcinoma and control mice. In control mice (n=8), the *Bub1* seems to be expressed more in the proximal colon (CPR) than in the distal colon (CD), however, in carcinoma mice (n=6), the expression seems equal between distal and proximal colon. Error bars represent the 95% confidence intervals.

6. DISCUSSION AND CONCLUSIONS

There is considerable evidence that the first changes towards colorectal cancer occur and can be detected in histologically normal tissue before the appearance of any detectable lesion (Hawthorn et al., 2014; Luo et al., 2014; Pussila et al., 2018). Furthermore, these changes may generate a field of tissue that is prone to malignant transformation, a phenomenon termed field defect. The aim of this thesis was to revise the previously reported gene expression changes in the mucosa next to the mouse proximal colon carcinoma and investigate whether these changes are also present further away from the carcinoma, in the distal colon. Fourteen mice, including six with proximal colon carcinoma, were selected for the study. The proximal and distal colon samples analysed in the present study were collected from each individual mouse.

The studied genes, *Bub1, Mis18a, Pms2, Rad9a, Tpx2*, and *Mlh1*, were selected based on the previously conducted genome-wide expression profiling, which revealed a significant downregulation of all the six genes in the carcinoma adjacent mouse colon mucosa (Pussila et al., 2018). All the studied genes are associated to functions that maintain the genomic stability. *Bub1* plays a role in the genomic stability maintenance by contributing to SAC function (V. L. Johnson et al., 2004). SAC is essential for proper chromosome alignment during the cell division, and its defects are associated with CIN (Jaffrey et al., 2000). Both *Tpx2* and *Mis18a* take part in the chromosome segregation. *Tpx2* contributes to microtubule organization and its alterations are associated with invasion and metastasis of colon cancer (Ping Wei et al., 2013). *Mis18a* is important for proper centromere function, and its loss causes severe defects in chromosomal segregation (Kim et al., 2012). *Rad9a* has many functions as it participates in several cell cycle checkpoints, apoptosis and DNA repair (Lieberman et al., 2011). *Mlh1* and *Pms2* are sufficient for the function of MMR mechanism (Jascur & Boland, 2006).

Suggestive evidence of the site-specificity of the expression changes in the carcinoma mice came from a previously conducted Master's thesis, where Rantamo (2017) investigated the gene expression in the distal colon of the mice and did not observe the gene expression changes that were previously observed in the proximal colon by Pussila et al. (2018). This observation seems reasonable since all the carcinomas were found in the proximal colon. However, the gene expression in the two colon parts were quantified by different techniques, proximal by RNA-seq and distal by TaqMan RT-qPCR, which is why the results are not comparable and cannot be used to reliably assess the site-specificity

of the gene expression levels. Here, in order to obtain comparable expression levels, both the proximal and distal colon samples were analysed with TaqMan RT-qPCR.

The expression levels of the six genes were compared between carcinoma and control mice in order to investigate whether the carcinoma status affected the overall gene expression levels in the colon. No significant expression differences for any of the genes were found between carcinoma and control mice on the general expression profiles (average of the two colon parts) (Fig. 9), or in the proximal colon mucosa alone (Fig. 10). The expression changes observed in the previous RNA-sequencing study of the carcinoma adjacent normal proximal colon mucosa were not detected in this study (Pussila et al., 2018). Although not statistically significant, *Rad9a* showed a reverse effect in the proximal colon, as the expression was slightly upregulated in the carcinoma adjacent normal mucosa in contrast to the RNA-seq study, where carcinomas were associated with the downregulated *Rad9a* expression. *Bub1*, however, seemed to have a downregulated trend in the mucosa of carcinoma mice, which is in line with the findings from the previous study. In distal colon, the expression level of most of the genes (*Bub1, Mis18a, Pms2, Rad9a,* and *Tpx2*) was not affected by the proximal colon carcinoma (Fig 11.). Interestingly, the *Mlh1* expression level in the distal colon was lower in the mice (p=0,046) that had carcinoma in their proximal colon than in the control mice (Fig 11). Considering the sample size and the fact that the detected difference was rather small, it is not clear whether the carcinoma status is the actual reason for the expression difference.

A previously reported increase in *Pms2* expression in the distal colon of the carcinoma mice was not detected in this study (Rantamo, 2017). We tried to reproduce the experiment with the cDNA used in the previous study in order to investigate the underlying reason for the distinct results. Interestingly, no difference was detected in the *Pms2* expression even though the exact same cDNA samples from the previous study were used (Fig. 12). Instead the results were perfectly in line with the primary results showed in Fig. 11, indicating that carcinoma status did not affect the *Pms2* expression in the distal colon.

It is known that some tumor characteristics tend to occur more frequently in certain colonic locations, such as MSI in the proximal colon tumors, which suggest that the proximal and distal colon mucosa may display distinct inherent or acquired features that makes it more susceptible to certain CRC pathways (Lee et al., 2015). Anatomically, the proximal and distal colon derives from different embryologic origins, as proximal colon originates from the midgut whereas the distal colon originates from the hindgut (Lee et al., 2015). Other differences that might affect the function and molecular

features of the proximal and distal colon mucosa are the varying diversity and concentration of microbes, and different exposure to bowel content (Lee et al., 2015). There are few previous studies where the gene expression patterns of the proximal and distal colon have been compared (Birkenkamp-Demtroder et al., 2005; Glebov et al., 2003). One study showed that over 1000 genes were differentially expressed in human proximal *versus* distal colon (Glebov et al., 2003). These genes were involved in several cellular functions, such as cell cycle control, proliferation, and DNA damage response and repair. Furthermore, the majority of the differentially expressed genes reported by Glebov et al. (2003) were expressed at higher level in the distal colon, suggesting that the distal colon has overall higher transcriptional activity than the proximal colon.

In order to investigate whether the expressions of the six studied genes have regional differences in healthy mouse colon, the expression profiles were compared between distal and proximal mucosa of control mice, that is, the mice without carcinoma (Fig. 13). No statistically significant differences were detected, which suggests that in normal condition, i.e. without cancer, the expression of the six studied genes is somewhat similar in the two mouse colon parts. Interestingly, *Bub1* seemed slightly downregulated in the distal colon of control mice. According to The Human Protein Atlas (www.proteinatlas.org), *BUB1* is expressed at higher level in the human sigmoid colon than in the transverse colon, which poses the question whether the downregulated trend of *Bub1* in the mouse distal colon might actually signify a true regional expression difference. Nevertheless, this is only speculation, and the issue requires further investigation with larger sample size before making any reliable conclusions.

One objective of the study was to investigate whether the proximal colonic mucosa adjacent to carcinoma displays different gene expression profile than the distal colonic mucosa further away from the carcinoma. The comparison of the proximal and distal colon gene expression levels in carcinoma mice did not reveal statistically significant differences, indicating that the carcinoma adjacent mucosa did not display site-specific gene expression changes (Fig. 14). When we took a closer look at the proportions of each gene within the carcinoma group and control group, we noticed an interesting trend in *Bub1* expression. In healthy control mice, *Bub1* seems to be expressed slightly more in proximal colon than in the distal colon (Fig. 13, Fig. 15), but this phenomenon was not observed in the carcinoma mice (Fig. 14, Fig. 15), indicating possible downregulation in the carcinoma adjacent mucosa. These findings suggests that *Bub1* downregulation may have a role in colon carcinogenesis, which is in line with the fact that mutations, epigenetic inactivation and aberrant expression of *Bub1* have been associated with chromosomally unstable CRC (Jaffrey et al., 2000; Pussila et al., 2018).

The results of the present gene expression analysis partly differ from those reported previously. There are several possible reasons for the distinct results. Especially with RT-qPCR, a poor reproducibility of the results seems to be a common problem (Bustin, Nolan, 2017). A major pitfall of RT-qPCR, or any cDNA quantification method, is the cDNA synthesis step. The cDNA yield from reverse transcription varies depending on the RT enzyme and the priming method, which is indeed observed in our results. The re-analysis of the *Pms2* expression was done with two cDNA sample sets, SR-2017 and SN-2018, that were synthesised with two different kits, RT^2 First Strand Kit (Qiagen) and SuperScript™ VILO™ cDNA Synthesis Kit (Thermo Fisher Scientific), respectively. Constantly higher Cq values were observed with the SR-2017 samples, indicating less efficient cDNA synthesis (Fig. 12).

RT-qPCR has long been the most widely used method to study gene expression, yet in recent years, the rapid development of novel sequencing and data-analysis methods have led to the increased use of RNA-sequencing. However, the robustness and reliability of RNA-seq methods in expression quantification are not completely clear. RT-qPCR and RNA-seq have their own pitfalls, nevertheless, both are considered as important techniques to study gene expression. The technique is typically selected based on the goals of the study, as RT-qPCR is a practical choice when studying the expression of few pre-defined genes whereas RNA-seq allows a whole-transcriptome profiling without prior knowledge of the transcript sequences (Bustin & Nolan, 2004). RT-qPCR was selected for the present study since the expressions of only six pre-defined genes were measured. Generally, a good concordance has been reported between RNA-seq and RT-qPCR results, which is why the obtained results were not consistent with our expectations (Su, Łabaj, Li et al., Nature Biotechnology, 2014). Even though the methodological issues might partially explain the differing results of the previously conducted RNA-seq study and the RT-qPCR study presented in this thesis, the most likely explanation for this is the small sample size. Here, the analysis was based on expression levels which were compared between the carcinoma and control group. The control group consisted of only eight non-carcinoma mice and showed relatively large within-group variation. The mean value of such a small number of samples is highly sensitive for errors arising from random individual differences (Slutsky, 2013). Therefore, the definition of the true control expression level requires much higher number of control samples. In RNA-seq experiment, where the carcinoma adjacent healthy mucosa was found to show significant gene expression changes, the control group consisted of 74 mice, and therefore, the mean expression values of the control group were presumably much closer to the actual gene expression levels of healthy mice.

In conclusion, the expression of *Bub1, Mis18a, Mlh1, Pms2, Rad9a* and *Tpx2* was studied in histologically normal colon mucosa from mouse distal and proximal colon. Altogether fourteen mice, of which six had proximal colon carcinoma, were selected for the expression analysis. Interindividual variation in the gene expression levels was rather wide among the mice, which complicated the reliable determination of mean expression values of the compared groups. No statistically significant gene expression differences were found between the carcinoma and healthy mice in this study, indicating that the studied mice did not display a cancer predisposing field in the carcinoma adjacent histologically normal colon mucosa. The results differed from the previously reported observations, where the expressions of the six studied genes were significantly downregulated in the carcinoma adjacent mouse mucosa when compared to the control mice. Both methodological and sampling related issues are likely to explain the inconsistent results. Although statistically non-significant, an interesting trend was noticed in *Bub1* expression pattern as the expression seemed slightly downregulated in the carcinoma adjacent mucosa. This phenomenon, as well as other suggestive evidence, should be addressed in future studies in order to identify biomarkers for early CRC development and further assess the relevance of the field defect concept.

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